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Studies on the *in vitro* propagation of *Calamus travancoricus*

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ABSTRACT

In vitro propagation techniques were applied for the mass propagation of economically important plant Calamus travancoricus Bedd.ex. Becc. & Hk.f. For callus induction, the explants were cultured on MS medium augmented with different auxins separately. The study showed 2, 4-D (10mg/ml) is effective to induce callus. The calli were subcultured on MS medium with various concentrations of cytokinins for shoot formation. The medium supplemented with BAP (5mg/l) showed better response. The medium supplemented with1.0mg/l of GA_3 was found suitable to culture microshoots for elongation. The in vitro shoots thus obtained were successfully rooted in ½ MS medium supplemented with IBA (1.5mg/l) and NAA (1mg/l) separately. The rooted plantlets were transferred to the field after acclimatization. Synthetic seeds were prepared.

Key words: *Calamus*, callus, organogenesis, multiple shoots, synthetic seed.
Abbreviations: ***BAP*** *- 6-Benzyl amino purine;* ***GA_3*** *- Gibberellic acid;* ***IBA*** *- Indole-3-butyric acid;* ***2,4-D*** *- 2,4-Dichlorophenoxy acetic acid;* ***NAA*** *- ά-Naphthalene acetic acid;* ***Kn*** *- Kinetin;* ***MS*** *- Murashige and Skoog's medium*

INTRODUCTION

The tissue culture work of Monocots is less known when compared to that of Dicots. This is especially true with reference to the family Arecaceae, commonly called the Palm family. The Arecaceae are a large family with about 212 genera and 3000 species [1]. In the family Arecaceae, the genus *Calamus* (commonly called cane or rattan) is considered economically very important. Rattans are extensively used for making furniture, fancy articles etc. Rattan products play an important role in the economic activity of many countries. In South East Asia, it is estimated that over 5 million peoples are involved in rattan industry. The annual global revenue exceeds US $ 6.7 billion. This cottage industry is highly labour-intensive and hence acts as a good employment generator. Rattans are also used in Ayurvedic system of medicine for curing various diseases like Cough, Edema, Herpes, Diabetes, Rabies etc [2]. *Calamus* oil extracted from the roots contains palmitic acid, iso-eugenol, calamine, calamol, etc. and is used in perfumery and for flavouring liquors. The ripe fruit pulp of *C. floribundus* is edible and is a dyspepsiac. The tender shoots of *C. erectus*, *C. floribundus* and *C. latifolius* are eaten as vegetable and also as a cure for stomach ulcer and muscular sprain [3].

Calamus is a paleotropical genus with about 370 species distributed throughout the world [4]. They are mainly found in the tropical rain forests and constitute an integral part of the tropical forest ecosystem. In Karnataka, 14 species of rattans have been recorded of which 5 species are endemic [5, 6]. In Karnataka, rattans are distributed in tropical wet evergreen forests and semi-evergreen to moist deciduous forests of Western Ghats from almost sea level to 1500m altitude with rainfall ranging from 1500-3000mm. Rattans are perennials, generally clustered, high

climbing spiny palms. They are dioecious, flowering is annual and pleonanthic. In most of the species of Indian rattans, the flowering is generally starts between October-January and fruits mature between April-June.

Over exploitation and deforestation from the past few decades have resulted in a drastic depletion of rattan resources in Karnataka. *Calamus rheedii* formerly reported in these forests could not be relocated in a past 10 decades. Probably this species is extinct or may be on the way to extinction [2]. The Red data book has already warned about *Calamus nagbettai*. A few species like *Calamus dransfieldii, C. karnatakensis, C. lakshmanae, C. prasinus, C. stoloniferus, C. travancoricus, C. vattayila* have decreased in their abundance in our forests. Although *C. hugelianus* is reported in 3 districts of Karnataka, now a days, it is becoming rare in the Karnataka forests. Under such circumstances, it is necessary to conserve their germ plasm. Despite this, it is surprising that, canes have not much attracted any studies. This may be because of their spiny character, inaccessibility and the difficulties in collecting flowers and fruits in forests at great heights [2].

Earlier literature on their tissue culture of Arecaceae shows that much work needs to be done. Only a few workers have been able to do some tissue culture of a few species of palms. The tissue culture of thousands of species of palms has remained uninvestigated. This is especially so with reference to the rattans, there is very little scientific information with reference to their tissue culture [7,8,9,10,11,12,13,14,15] These works have established that it is possible to propagate rattan through tissue culture. Therefore, active research is needed to understand the tissue culture behaviour of these interesting climbers to save some of them from extinction.

In nature, rattans are generally propagated by seeds. The main problem in this method of propagation is production of quality seeds. Because rattans are dioecious, when a fruiting female plant is extracted the supply of seed is affected. Seed viability and germinability are low. Most seeds do not germinate if stored for long and this has also in a way led to the scarcity of planting material.

It is in this background that tissue culture multiplication of rattan gains importance as an alternative method for propagation. In view of the above, to fill the gap in our knowledge on the tissue culture of Rattans, the present studies were undertaken.

MATERIALS AND METHODS

The materials for the present investigation included cabbage leaf, mature fruits and terminal bud portion of *C. travancoricus* (Fig.1A), were collected from Makutta (Kodagu district) forest areas in the Western Ghats of Karnataka.

Collection of embryo

The scaly pericarp and the fleshy sarcotesta of mature fruits are first removed to obtain the hard seeds. The seeds are washed in water and then with 0.1% Tween-20 for three minutes, followed by washing in 10% chlorox for about 10 minutes. The seeds are then washed several times in sterile distilled water to remove all traces of chlorine. The embryo is located in a cavity immediately below the operculum. The embryo is taken out carefully by removing the endosperm tissues around it.

Collection of collar region from seedlings

The collar region was collected from *in vitro* germinated seedlings from seeds on MS medium without plant growth regulators. The radicle was cut off along with one or two outer-layers of sheath and the top part of inner-sheath and the collar was collected.

Collection of terminal bud

Terminal buds of rattan are deeply located at the stem tip. Bud explants were collected with sheaths and a segment of 40-60 cm from the tip was cut to ensure the inclusion of terminal bud. Cabbage-leaves were also included in bud explant.

Sterilization of explants

The explants were thoroughly washed under tap water and rinsed with 0.1% Tween–20 detergent for 5-10 minutes followed by washing under running tap water for 15-20 minutes. The explants were then treated with 0.4% Bavistin

solution for 2-3 minutes followed by washing 3-4 times with sterile distilled water. They were then treated with 0.1% mercuric chloride solution for 3-5 minutes and rinsed thrice in sterile distilled water.

Synthetic seed preparation

The sterilized explants were blotted to dryness on a sterile filter paper. Explants were then mixed in different concentrations of sodium alginate gel (2-7%) prepared in full strength MS basal medium supplemented with 3% sucrose. After thorough mixing, the explants were slowly dropped into a sterile solution of $CaCl_2$. $2H_2O$ (1.36g/150ml), allowed to form beads and were retained in this complexing agent for ½ hr. The encapsulated explants were thoroughly rinsed 3-4 times with sterile MS liquid medium to remove traces of $CaCl_2$. The alginate beads were then collected and transferred to sterile filter paper placed in petridishes. Blot dried beads were stored for 6-12 months at 4^o C

Culture media

The sterilized explants were cultured on Murashige and Skoog (MS) [16], B_5 [17] and White's [18] media containing different concentration and combinations of auxins and cytokinins. The result obtained on MS medium with slight modification was better than other media used. For all the experiments, MS medium supplemented with 3% sucrose was used. For callus induction the medium is supplemented with NAA (0.5-4.5mg/l), IBA (0.5-5.5 mg/l) and 2, 4-D (1.0-11.0mg/l) separately. For shoot induction, the callus obtained from each explant was separated in to smaller units and sub-cultured on to the medium supplemented with BAP (1.0-6.0mg/l), KN (0.5-6.0mg/l) and in combination of BAP & KN (1.0-3.0 + 0.2-1.0mg/l) separately. Microshoots obtained in various medium compositions were transferred to shoot elongation medium comprising MS medium supplemented with GA_3 (0.5-2.0mg/l). For root induction, the mature shoots of 2cm height were separated and cultured in MS medium supplemented with IBA (0.1-2.0mg/l) and NAA (0.1-2.0mg/l) separately. The pH of the medium was adjusted to 5.8 followed by the addition of 0.8% agar before autoclaving. The medium was melted and dispensed in to culture tubes and bottles followed by autoclaving at 15 psi for 15-20 minutes at 121°C. The culture tubes and bottles were maintained in the culture room at 27 ± 2 °C under a 10-12hr photoperiod. The illumination was provided by cool white fluorescent bulbs with the light intensity ranging from 1000 to 1500 Lux.

Acclimatization

For hardening, the *in vitro* rooted plants were transferred to pots containing pearlite-clay soil-farm yard manure (3:6:1 by volume). The soil mixture was moistened with reduced concentration of (half concentration) of MS salts and vitamins. Initially high humidity was maintained by covering the pots with polythene bags. After two weeks, polythene bags were removed and they were exposed gradually to sunlight for their acclimatization. They were finally transferred to pots containing garden soil.

Statistical Analysis

Five replicates were used per treatment and the entire experiment was repeated thrice to confirm the results. The data for frequency of caulogenesis, multiple shoot induction and root induction for all the explants were recorded after 4-6 weeks of culture. The standard error was calculated for each mean and it was further subjected to analysis of variance.

RESULTS AND DISCUSSION

Experiments were aimed at obtaining *in vitro* plantlets from various explants of *Calamus travancoricus*. Among the explants tried such as young shoot buds, cabbage leaves, collar region from seedlings and embryo, the latter two explants i.e., collar region from seedlings and embryo were found to be good for initiation of callus and multiple shoots in MS medium. As in other palms the browning of tissue during the early period of incubation was heavy and autocatalytic. To minimise the browning problem, various methods were tried. Addition of charcoal, ascorbic acid, polyvinylpyrrolidone, cysteine and maintaining cultures in darkness was not effective. The most effective method was frequent subculture. The explants were transferred every alternate day to fresh medium for more than one week to avoid browning of tissue.

For callus induction, the explants were cultured on MS medium augmented with NAA (4mg/l), IBA (5mg/l) and 2, 4-D (10mg/l -Table 1) separately. In all the cases callus induction was observed but the percentage of caulogenesis frequency was varied. In the media supplemented with NAA maximum caulogenesis frequency observed was 62%. The IBA supplemented media showed 63%. Like wise, in 2, 4-D augmented media the frequency of caulogenesis

observed was and 91% (Table 1). The study clearly showed that, 2, 4-D is highly effective to induce callus efficiency in the presently investigated species of *Calamus*. Calli were initiated within 15-20 days of inoculation (Fig.1B). The period involved for complete development of callus was 30-40 days. Calli could not be initiated in the medium without growth regulators. ln an initial investigation Umali-Garcia [8] examined 11 species of *Calamus* and two of *Daemonorops*.The explants used were from the shoot apex region (cabbage). In three species of *Calamus*,the explants developed callus which subsequently gave rise to shoots. These were isolated and rooted in a different medium to obtain complete plantlets. On the other hand Padmanabhan and Krishnan [19] and Padmanabhan and Sudhersan [20] only obtained an increase in size of the leaf explants and formation of a wrinkled mass of laminate tissue which they termed as 'laminoid'. Rao [21] reported role of different concentration of auxins and cytokinins in the *in vitro* propagation of different species of *Calamus*. Attempts have been made to study growth and development of various explants of rattans under aseptic conditions. Yusuff and Manokaran [22] cultured the root and shoot tips of *Calamus manan.* They obtained the friable callus that became nodular and opaque, and embryogenesis was observed. Chuthamas *et al*. [23] obtained normal seedlings from immature embryos of *C. manan* on MS and Y3 media. Dekkers and Rao [13] cultured embryos of *C. trachycoleus* on MS medium. After 8 weeks of inoculation, the collar region was covered with callus and green shoots.

The calli were subcultured on MS medium with various concentrations of cytokinins for shoot formation. The duration for proliferation after subculture was 35-40 days. The medium supplemented with BAP alone (5mg/l) showed better response i.e., 82-87%. The number of shoots produced was 4-5 and the shoot length varied from 1.7-2.0cm (Table 2). The medium augmented with Kinetin alone (4mg/l) induced 2-3 shoots, the mean shoot length observed was 1.4-1.65cm and the shoot inducing response was about 65% (Table 3). The callus also responds well to MS medium supplemented with BAP and Kinetin in combination (3+0.5mg/l). These cultures later gave rise to 3-4 shoots per culture (Table 4). It was observed that 3-5mg/l BAP alone was better for both shoot multiplication as well as shoot elongation for the presently investigated species of *Calamus*. Kinetin alone was not much effective for shoot multiplication but in combination with BAP which gave better result (Table 2, 3 & 4). Eeuwens [24] reported that when immature embryos of *Calamus manan* were cultured on MS and Y3 media, these were able to grow into complete plantlets. Barba *et al*. [9] cultured the embryos of *Calamus manillensis* and obtained shoots. These could be excised and rooted to obtain complete plantlets. Gunawan & Yani [11] cultured embryos of *Calamus manan* on MS media. The embryo was induced to produce callus which subsequently could differentiate several plantlets. The plantlets were separated and transferred to soil and then to the field. Yusoff [12] succeeded in inducing multiple shoots from the collar region of the *in vitro* raised seedlings of *Calamus manan* on MS + BAP or Kn (10^{-6} to $l0^{-4}$ M). The multiple shoot structure could be subdivided and subcultured onto a medium with a lower cytokinin concentration. The rooted plantlets were subsequently transferred to soil.

The *in vitro* produced shoots were exposed to MS medium fortified with various concentrations of GA_3 for further elongation. After 4 weeks, medium supplemented with1.0mg/l of GA_3 was found suitable to culture microshoots for elongation. This appropriate medium composition enhanced the microshoots to grow up to 2.7 cm in height with 84.3-88.3% of shoot elongation response in the presently investigated species of *Calamus*. Lower concentration of GA_3 (0.5mg/l) caused slight elongation of microshoots. There was no significant elongation of microshoots at higher concentration of GA_3 (2mg/l) (Table 5). The same has been reported by Zeng Bingshan [25] in different species of rattans.

The elongated shoots were transferred to 1/2MS medium containing auxins for rooting at various concentrations. Rooting was observed in ½ MS medium augmented with IBA (1.5mg/l) and NAA (1mg/l) separately (Table 6 & 7) (Fig. 1C). The shoots of 2-2.5cm long were found to be suitable for the purpose of rooting. Roots were observed after 28 days of inoculation. Rooted plantlets on agar solidified media were removed and subjected to different modes of hardening and acclimatization and finally transferred to the soil. Pioneering work on cane micropropagation has been done in Philippines and Malaysia. Complete plantlet regeneration through consistently reproducible protocols has been reported for *C. manillensis*, *C. manan* and *C. rotang* [3]. The same has been studied by Zeng Binghsan [25] in different species of rattans.

Synthetic seeds were prepared for conservation of germplasm using different explants with different concentration of sodium alginate (Fig. 1D). Of the different concentrations of sodium alginate used, an optimal ion exchange between Na^+ and Ca^{2+} produced firm, clear beads at3% sodium alginate solution The beads were too soft to handle a lower concentrations of sodium alginate, but there was lesser percentage of hyperhydricity. However, the beads were too hard at higher concentrations, which delayed shoot emergence.

The present study clearly showed that, the regenerating potential of different explants of *C. travancoricus.* Of the explants used, the embryo and collar region of seedlings gave better result when compared to terminal bud and cabbage leaves. The rate of callus induction observed in embryo and collar region of seedlings was 81-86% and 72-78% respectively. Like wise, the rate of shoot induction observed in case of embryo and collar region of seedling was 96-98% and 92-95% respectively.

Table 1. Effect of NAA, IBA and 2, 4-D in MS medium on callus induction in *C.travancoricus*

NAA (mg/l)	Caulogenisis frequency(%)	IBA (mg/l)	Caulogenisis frequency(%)	2,4-D(mg/l)	Caulogenisis frequency(%)
Control	00	Control	00	Control	00
0.5	8	0.5	10	1.0	13
1.0	12	1.0	11	2.0	18
1.5	20	1.5	13	3.0	26
2.0	35	2.0	18	4.0	35
2.5	42	2.5	21	5.0	41
3.0	51	3.0	26	6.0	54
3.5	54	3.5	32	7.0	60
4.0	**62**	4.0	38	8.0	65
4.5	49	4.5	51	9.0	74
		5.0	**63**	**10.0**	**91**
		5.5	41	11.0	63

Table 2. Effect of BAP in MS medium on shoot induction in *C. travancoricus*

BAP (mg/l)	Response (%)	Mean shoot number	Mean shoot length (cm)
Control	00	00	00
1.0	24.7 ± 0.41	0.8 ± 0.08	0.12 ± 0.03
1.5	26.0 ± 0.3	1.9 ± 0.35	0.49 ± 0.04
2.0	38.7 ± 0.37	2.4 ± 0.51	0.96 ± 0.01
2.5	41.3 ± 0.78	3.3 ± 0.33	1.5 ± 0.14
3.0	45.0 ± 0.4	3.8 ± 0.65	1.56 ± 0.55
3.5	52.5 ± 0.84	3.6 ± 0.17	1.49 ± 0.13
4.0	63.1 ± 0.28	4.3 ± 0.44	1.66 ± 0.24
4.5	69.4 ± 0.9	4.6 ± 0.99	1.72 ± 0.2
5.0	**86.8 ± 1.5**	**5.1 ± 1.1**	**1.99 ± 0.12**
5.5	59.5 ± 0.73	3.2 ± 0.6	1.43 ± 0.24
6.0	34.3 ± 0.98	1.9 ± 0.09	0.98 ± 0.06

Table 3. Effect of Kinetin in MS medium on shoot induction in *C. travancoricus*

Kinetin (mg/l)	Response (%)	Mean shoot number	Mean shoot length (cm)
Control	00	00	00
1.0	18.3 ± 0.14	0.76 ± 0.09	0.2 ± 0.01
2.0	29.5 ± 0.52	1.27 ± 0.1	0.47 ± 0.07
3.0	51.01 ± 0.26	2.1 ± 0.26	0.83 ± 0.04
4.0	**63.2 ± 0.6**	**3.0 ± 0.06**	**1.65 ± 0.04**
5.0	36.2 ± 0.23	1.9 ± 0.11	1.1 ± 0.14
6.0	16.24 ± 0.32	0.63 ± 0.08	0.33 ± 0.06

Table 4. Effect of BAP & Kinetin combination in MS medium on shoot induction in *C.travancoricus*

BAP + Kientin (mg/l)	Response (%)	Mean shoot number	Mean shoot length (cm)
Control	00	00	00
1.0 + 0.2	13.3 ± 0.08	0.2 ± 0.01	0.1 ± 0.02
1.0 + 0.5	22.1 ± 0.1	0.7 ± 0.04	0.3 ± 0.01
1.0 + 1.0	21.3 ± 0.2	0.7 ± 0.05	0.25 ± 0.02
2.0 + 0.2	41.4 ± 0.23	1.1 ± 0.21	0.4 ± 0.08
2.0 + 0.5	49.3 ± 0.33	1.7 ± 0.1	0.7 ± 0.05
2.0 + 1.0	51.2 ± 0.16	2.3 ± 0.21	1. 1 ± 0.09
3.0 + 0.2	68.1 ± 0.25	2.9 ± 0.4	0.23 ± 0.12
3.0 + 0.5	**74.3 ± 0.91**	**3.7 ± 0.21**	**1.66 ± 0.07**
3.0 + 1.0	55.0 ± 0.31	1.8 ± 0.2	0.8 ± 0.15

Table 5. Effect of Gibberellic acid (GA_3) on elongation of microshoots of *C. travancoricus* cultured on MS medium

GA_3 (mg/l)	Shoot elongation (%)	Mean shoot length (in cm)
Control	17.2 ± 0.3	0.7± 0.11
0.5	65.1 ± 0.11	1.38 ± 0.31
1.0	**88.3 ± 0.33**	**2.7± 0.4**
1.5	65.2 ± 0.27	1.62± 0.2
2.0	31.2 ± 0.14	1.0 ± 0.09

Table 6. Effect of IBA in ½ MS medium on rooting of *in vitro* shoot of *C. travancoricus*

IBA(mg/l)	Response (%)	Mean root number	Mean root length (cm)
Control	00	00	00
0.1	18.6 ± 0.09	2.1 ± 0.02	0.66 ± 0.02
0.5	37.2 ± 0.11	4.8 ± 0.24	1.1 ± 0.04
1.0	55.1 ± 0.2	8.6 ± 0.29	1.94 ± 0.03
1.5	**88.1 ± 0.3**	**15.4 ± 0.14**	**2.91 ± 0.05**
2.0	40.2 ± 0.4	5.7 ± 0.21	1.12 ± 0.03

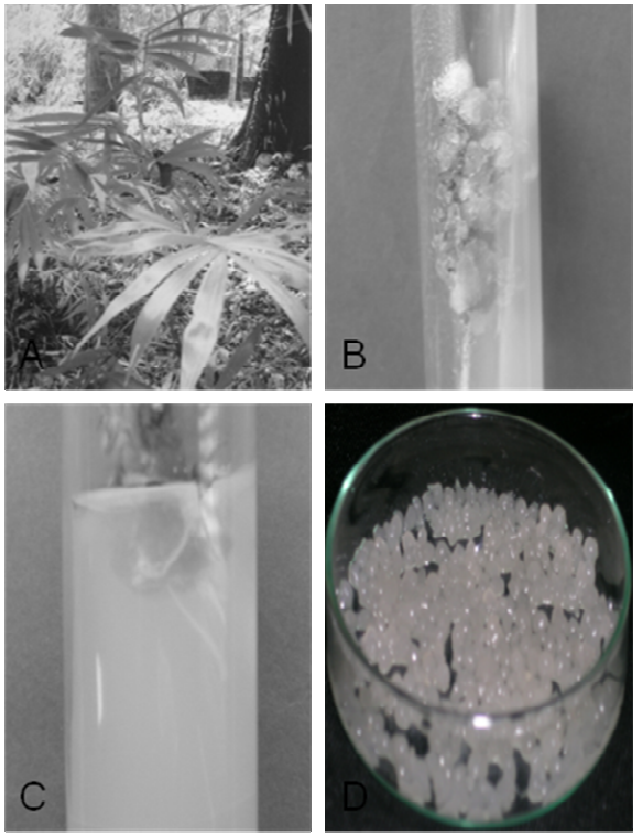

Fig. 1 A-D - A. *Calamus travancoricus*. **B.** Shows appearance of callus. **C.** In vitro rooting of microshoots. **D.** Shows explants encapsulated in 3% sodium alginate solution.

Table 7. Effect of NAA in ½ MS medium on rooting of *in vitro* shoot of *C. travancoricus*

NAA(mg/l)	Response (%)	Mean root number	Mean root length (cm)
Control	00	00	00
0.1	28.6 ± 0.09	3.1 ± 0.02	0.68 ± 0.02
0.5	47.2 ± 0.12	7.8 ± 0.04	1.19 ± 0.04
1.0	**85.1 ± 0.24**	**13.6 ± 0.29**	**2.84 ± 0.04**
1.5	48.1 ± 0.31	5.4 ± 0.14	1.29 ± 0.05

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